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Date • March 31, 2009

Pages • 20

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Transmit To • Ms. Michelle Williams

Company/Firm • Publications - Certificate of Correction Branch
U.S. Patent and Trademark Office

Telephone No. • 703.308.9390 x 118

Fax No. • **571.270.9945**

From • Judith A. Sherman

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Attorney• Stephanie Seidman, Reg. No. 33,779

Reference / Subject • **Replacement of Request for COC filed 02/13/2009**
US Patent No. 7,329,728 – USSN 09/586,625
Ligand Activated Transcriptional Regulator Proteins

Client ID/Matter No. • 0119362-00002 / 1227B

COMMENTS: As requested, attached is a replacement for the missing ***Request for Certificate of Correction*** filed 02/13/2009 which includes the *Transmittal Letter*, *Request for Certificate of Correction* (2pgs), *Form PTO/SB/44* (1pg), supporting documents (14 pgs), along with the return post card reflecting the OIPE date stamp of FEB 13 2009.

Your assistance in getting this Request into the system and reviewed is very much appreciated.

Judy

When you are sending to us, please be sure to include a cover sheet with your transmittal and a telephone number where you can be contacted in case of equipment malfunction.

Transmitted by: **Time:**

Attorney's Docket No. 119362-00002/1227B	Express Mail Label No. EM 247737712 US	Mailing Date February 13, 2009	*For PTO Use Only Do Not Mark in This Area*
Application No. 09/586,625	Filing Date June 2, 2000	Attorney/Secretary Init SLS/KCM/prp	
Patent No. 7329728	Issue Date February 12, 2008		
Title of the Invention LIGAND ACTIVATED TRANSCRIPTIONAL REGULATOR PROTEINS			
Applicant Harbus III *et al*			

Enclosures

- Transmittal Letter (1 page in duplicate);
- Request for Certificate of Correction (2 pages).
- Supporting Documents (14 pages),
- Certificate of Correction Substitute Form PTO/SB/44 (1 page); and
- This Return Postcard

Attorney Docket No.: 119362-00002/1227B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Barbas III *et al.*	Art Unit	: 1652
Patent No.	: 7,329,728	Examiner	: Lorraine Spector
Issue Date	: February 12, 2008	Conf. No.	: 6568
Serial No.	: 09/586,625	Cust. No.	: 77202
Filed	: June 2, 2000		
Title	: LIGAND ACTIVATED TRANSCRIPTIONAL REGULATOR PROTEINS		

Attn.: Certificate of Correction Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

TRANSMITTAL LETTER

Dear Sir:

Transmitted herewith are a Request for a Certificate of Correction pursuant to C.F.R. § 1.322 & 1.323 (2 pages), supporting documents (14 pages), Certificate of Correction Form PTO-1050 (1 page), and a return postcard for filing in connection with the above-identified application. Since the errors appear to be those of the Patent Office, no fee should be due. If it is determined that a fee is due, the Office is hereby authorized to charge the fee to Deposit Account No. 02-1818.

☒ The Commissioner is hereby authorized to charge any fees that may be due in connection with this paper or with this application during its entire pendency to Deposit Account No. 02-1818. A duplicate of this sheet is enclosed.

Respectfully submitted,

Stephanie Seidman
Reg. No. 33,779

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CERTIFICATE OF MAILING BY "EXPRESS MAIL"
"Express Mail" Mailing Label Number EM 247737712 US
Date of Deposit February 13, 2009
I hereby certify that this paper is being deposited with the United States Postal "Express Mail Post Office to Addressee" Service under 37 CFR §1.10 on the date indicated above and is addressed to: Commissioner for Patents, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450.

Steven Dennis

APR 0 1 2009

Attorney Docket No.: 119362-00002/1227B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Barbas III *et al.*
Patent No. : 7,329,728
Issue Date : February 12, 2008
Serial No. : 09/586,625
Filed : June 2, 2000
Title : LIGAND ACTIVATED TRANSCRIPTIONAL REGULATOR PROTEINS

Art Unit : 1652
Examiner : Lorraine Spector
Conf. No. : 6568
Cust. No. : 77202

Attn.: Certificate of Corrections Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION

Dear Sir:

Pursuant to 37 C.F.R. § 1.322, the patentee respectfully requests reconsideration of one item of a Request for a Certificate of Correction, originally submitted on May 2, 2008.

IN THE TITLE PAGES:

In Item (56) References Cited, in OTHER PUBLICATIONS:

please add the following reference: — Blau et al., "γ-globin gene expression in CID-dependent multi-potential cells established from beta-YAC transgenic mice," J. Biol. Chem. Papers in Press. Published August 30, 2005 as Manuscript M504402200—.

CERTIFICATE OF MAILING BY "EXPRESS MAIL"
"Express Mail" Mailing Label Number EM 247737712 US
Date of Deposit February 13, 2009
I hereby certify that this paper is being deposited with the United States Postal "Express Mail Post Office to Addressee" Service under 37 CFR §1.10 on the date indicated above and is addressed to: Commissioner for Patents, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA, 22313-1450.

Steven Dennis

APR 0 1 2009.

Applicant : Barbas et al.
Patent No. : 7,329,728
Issued : February 12, 2008
Serial No. : 09/586,625
Filed : June 2, 2000

Attorney Docket No.. 119362-00002/1227B
Request for Certificate of Correction

REMARKS

A Certificate of Correction (Form PTO-1050) incorporating the above changes is included with this Request. Because it appears that this second request for a Certificate of Correction is necessary because of an error on the part of the Patent Office, no fee should be due. If it is determined that a fee is due, the Office is hereby authorized to charge any fees due herein to Deposit Account No. 02-1818.

This Certificate of Correction seeks to correct an omission by the PTO in the "OTHER PUBLICATIONS" sections of the References Cited, Item (56). This reference was provided to the Patent Office on a PTO-1449 form on November 22, 2005. The reference provided to the Patent Office was a "Paper in Press" and therefore does not have a volume number nor page numbers. The identifying information, indicated at the top of the first page, states: "JBC Papers in Press, published on August 30, 2005 as Manuscript M514402200" along with the Authors and Title. Therefore as provided on PTO-1449 form, the Blau et al. reference includes this identifying information: Author, Title, Journal and the date August 30, 2005. This reference was considered by the examiner, as evidenced by the examiner-initialed PTO-1449 form, mailed to the Applicant on August 7, 2006. Hence this reference was entered into the filing history of the application and should appear on the face of the patent. A copy of the examiner-initialed PTO-1449 form, mailed to the Applicant on August 7, 2006, and a copy of Blau et al., as provided to the Patent Office, are attached.

Patentee respectfully requests correction of this error by issuance of a Certificate of Correction.

Respectfully submitted,

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SUBSTITUTE FORM PTO/SB/44 (5-03)

Staple Here Only

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. .: 7,329,728
APPLICATION NO .: 09/586,625
DATED .: FEBRUARY 13, 2009
INVENTOR(S) .: BARBAS III ET AL.

It is certified that an error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

IN THE TITLE PAGES:

In Item [56] References Cited, in OTHER PUBLICATIONS:
please add the following reference: — Blau et al., "γ-globin gene expression in CID-dependent multi-potential cells established from beta-YAC transgenic mice," J. Biol. Chem. Papers in Press. Published August 30, 2005 as Manuscript M504402200—

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APR 0 1 2009

JBC Papers in Press. Published on August 30, 2005 as Manuscript M504402200

γ-GLOBIN GENE EXPRESSION IN CID-DEPENDENT MULTI-POTENTIAL CELLS ESTABLISHED FROM β-YAC TRANSGENIC MICE*

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Running title: CID-dependent β-YAC bone marrow cells

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Identification of trans-acting factors or drugs capable of reactivating γ-globin gene expression is complicated by the lack of suitable cell lines. Human K562 cells co-express ε- and γ-globin, but not β-globin; transgenic mouse erythroleukemia 585 cells express predominantly human β-globin, but also γ-globin; and transgenic murine GM979 cells co-express human γ-and β-globin. Human β-globin locus yeast artificial chromosome transgenic mice display correct developmental regulation of β-like globin gene expression. We rationalized that cells established from the adult bone marrow of these mice might express exclusively β-globin, and therefore could be employed to select or screen inducers of γ-globin expression. A thrombopoietin receptor derivative that brings the proliferative status of primary mouse bone marrow cells under control of a chemical inducer of dimerization was employed to institute and maintain these cell populations. Human β-globin was expressed, but γ-globin was not; a similar expression pattern was observed in cells derived from fetal liver. γ-globin expression was induced upon exposure to 5-azacytidine, in cells derived from -117 Greek hereditary persistence of fetal hemoglobin β-YAC mice, showing that the HPFH phenotype was maintained in these cells, or was reactivated by an artificial zinc-finger-γ-globin transcription factor and the previously identified fetal globin transactivators FKLF and FGIF. These cells may be useful for identifying transcription factors that reactivate γ-globin synthesis or screening γ-globin inducers for the treatment of sickle cell disease or β-thalassemia.

Members of the human β-like globin gene family are developmentally regulated. The genes are arrayed in the order in which they are expressed during development, 5'-ε-^{G}γ-^{A}γ-δ-β-3'. During primitive erythropoiesis, the embryonic ε-globin gene is expressed in nucleated, yolk-sac derived erythroid cells. Later, during fetal definitive erythropoiesis, the tandem fetal γ-globin genes are expressed in enucleated erythroid cells of the fetal liver. Finally, the β-globin gene, and to a much lesser extent the δ-globin gene, are expressed initially in the liver during fetal definitive erythropoiesis and ultimately in bone marrow-derived erythroid cells during adult definitive erythropoiesis. The temporally regulated expression of the β-like globin genes provides a paradigm for developmental control in mammalian cells. Transgenic mice have been instrumental to the identification of many *cis*-acting elements and trans-acting factors necessary for normal developmental control. The individual globin genes, including their gene-proximal regulatory sequences, are variably expressed in transgenic mice. However, when linked to the locus control region (LCR)[1], a powerful regulatory motif consisting of five DNAseI-hypersensitive sites (HSs) located upstream of the ε-globin gene, high-level, copy number-dependent, integration site-independent expression is achieved. Correct human hemoglobin switching can be largely mimicked in transgenic mice containing a human β-globin locus yeast artificial chromosome (β-YAC; 1). Although mice do not have a fetal stage

of globin gene expression, the human γ-globin genes are expressed in the fetal liver, similar to humans.

An improved understanding of globin gene regulation is clinically relevant, since the beneficial effects of elevated fetal hemoglobin levels (HbF, $\alpha_2\gamma_2$) in patients with sickle cell anemia and β thalassemia are well documented (2). Considerable attention has been directed at identifying krüppel-like factors (KLFs) that specifically transactivate γ-globin gene expression as a potential approach to gene therapy (3, 4). Additionally, many investigations have focused on identifying new pharmacological compounds that are capable of inducing γ-globin production (5). However, the identification of γ-globin inducers, either proteins or drugs, has been hampered by the lack of suitable *in vitro* model systems for selection of activators or for screening chemical compounds.

A number of *in vitro* models for evaluating putative γ-globin inducers have been reported. Cultures of primary adult human erythroid progenitors capitalize on the significant levels of γ-globin that are detected in these cells. Cultures of primary human burst forming units-erythroid (BFUe), either in clonogenic assays (6) or in suspension (7) have been used to evaluate putative γ-globin inducers. However, these assays cannot be standardized; thus they are difficult to use for large scale screening. While greater levels of standardization can be achieved with established erythroid cell lines, none of the human erythroleukemia lines display a normal adult pattern of globin gene expression. K562 cells, commonly used for this purpose, predominantly express γ-globin, variable levels of ε-globin, and no β-globin. Therefore, an increase in γ-globin gene transcription in K562 cells may be a consequence of promoting globin gene expression generally, rather than via a mechanism that preferentially activates γ-globin gene expression. Mouse erythroleukemia (MEL) cells display an adult pattern of globin gene expression; however, as noted above, mice lack a fetal globin gene. Initial attempts to develop MEL cells suitable for screening γ-globin inducers involved the generation of stably transfected cells incorporating various DNA fragments containing the human γ-globin gene, but transgenic cell lines generated in this manner failed to demonstrate proper regulation of the introduced γ-globin gene (8). Similarly, γ-globin gene expression in MEL cells containing a human β-globin locus YAC transgene was not correctly regulated (9, 10). In MEL 585 cells, β-globin gene expression predominates, although some γ-globin gene product is observed, whereas in GM 979 cells γ-globin and β-globin are co-expressed. Finally, developmental stage appropriate expression of human globin genes is achieved over time in hybrids generated from MEL cells fused to human fetal liver cells (HFE-MEL hybrids) or in MEL cells fused to human lymphoblasts (11). However, the degree of completion of switching is variable and the cells eventually display the globin expression pattern of the terminal MEL cells used in the initial fusion.

None of the aforementioned cell systems completely mirrors adult erythropoiesis; that is, γ-globin expression is markedly higher than normal adult human physiological levels, even in those cell lines where β-globin is the major species synthesized. Thus, these models can only be used to screen for enhancement of all ready existent low-level γ-globin gene expression, rather than select for activation of a silent γ-globin gene. We reasoned that immortalized cells derived from the bone marrow of β-YAC transgenic mice might express exclusively β-globin, since the human pattern of globin transgene synthesis was recapitulated in these mice. β-YAC bone marrow cells (BMCs) were established using an artificial proliferation signal comprised of the thrombopoietin (mpl) signaling domain fused to FKBP binding domains responsive to a chemical inducer of dimerization (CID; 12-14). In the presence of a CID, homodimers are generated and the resultant growth signal maintains the BMC population indefinitely as long as the CID is present. These cells exclusively express human β-globin, but γ-globin expression can be reactivated by various treatments or through the presence of a hereditary persistence of fetal hemoglobin (HPFH) mutation in the ^γ-globin gene. Therefore, these CID-dependent, multi-potential β-YAC BMCs provide a model system in which γ-globin protein transactivators can be selected and pharmacologic inducers of HbF definitively screened.

EXPERIMENTAL PROCEDURES

Transgenic mice. Generation of β-YAC transgenic mice was described previously (1). *Ppo*-155 β-YAC transgenic mouse line 1, containing the 155 Kb β-YAC (15), or -117 β-YAC transgenic mice, containing the 248 Kb -117 $^{A}\gamma^{m}$ Greek HPFH β-YAC (16), were the sources of bone marrow or fetal liver used to establish CID-dependent cell populations.

Derivation of drug-dependent, multi-potential cells and cell culture. CID-dependent cells were derived as described previously (14, 17). Briefly, 5- fluorouracil (5-FU; 150 mg/kg) was injected intraperitoneally into 155 Kb wild-type β-YAC transgenic mice. After 48 hours, marrow cells were collected and cultured for 48 hours in Dulbecco's Modified Eagle Medium (DMEM) containing 16% fetal calf serum (Hyclone; Logan, UT), 5% mouse IL-3 supplement (BD, Bedford, MA), rhIL-6 (100 ng/ml) and rmSCF (50 ng/ml; Chemicon, Temecula, CA) at 37°C in 5% CO_2. After pre-stimulation, cells were transferred onto irradiated (1,500 cGy) GPE+86 producer cells containing a F36V-modified FKBP12 derivative fused to the intracellular portion of the thrombopoietin receptor, mpl. Two F36Vmpl vectors were used, one containing a GFP marker downstream of an internal ribosomal entry site (IRES, 17), or a neo reporter expressed from a separate PGK promoter (14). Transductions were carried out using the same growth factors as for prestimulation with the addition of polybrene (8 μg/ml; Sigma-Aldrich, St. Louis, MO). After 48 hours, cells were washed and cultured in the presence of AP20187 dimerizer (100 nmol/l; Ariad Pharmaceuticals, Cambridge, MA) in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FCS, penicillin and streptomycin. CID-dependent BMCs were similarly established from 248 Kb -117 Greek HPFH β-YAC transgenic mice. This approach also was used to establish drug-dependent cells from transgenic murine fetal liver (day 12 post-conception). Livers were dissected from day 12 fetuses and single cell liver suspensions prepared as described (18).

Stable transfection of CID-dependent wild-type β-YAC BMCs with pcDNA3.1/Hygro gg1-VP64. A 0.8 Kb *ApaI-HindIII* (New England Biolabs, Beverly, MA) fragment was isolated from pcDNA-gg1-VP64-HA (19) and ligated into *ApaI-HindIII*-cut and phosphatased (calf intestinal alkaline phosphatase, Promega, Madison, WI) pcDNA3.1/Hygro (+) (Invitrogen, Carlsbad, CA) to produce pcDNA3.1/Hygro gg1-VP64 so that transfected cells could be selected for hygromycin resistance. CID-dependent cells derived from β-YAC mice (3.5×10^6) were washed in PBS and resuspended in 0.8 ml DMEM. During the transfection, both cells and the DNA/Lipofectamine (Invitrogen, Carlsbad, CA) mix were maintained in DMEM. The DNA/Lipofectamine mix was prepared according to the manufacturer's instructions using 4 μg plasmid DNA in 10 μl Lipofectamine; this mix was incubated for 45 minutes. Cells were added to the DNA/Lipofectamine and the transfection mixture was incubated for 6 hours at 37°C in 5% CO_2 before adding 3 ml IMDM containing 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 1X non-essential amino acids and 10 mM HEPES supplemented with CID and 0.1 ml extra FBS. After overnight incubation, cells were centrifuged (200 x g, room temperature) and resuspended in IMDM supplemented with CID and 200 μg/ml hygromycin. After selection, clones were generated by limiting dilution and were screened by Southern blot hybridization analysis (data not shown).

Enforced expression of potential fetal globin transactivators in CID-dependent wild-type β-YAC BMCs. Enforced erythroid-specific expression of cDNAs may be obtained by cloning them into the unique *BglII* restriction enzyme site of a derivative of our expression vector, pμ'LCR-β pr-*BglII*-β int2-enh (20, 21). Full-length cDNAs of human NF-E4 (550 bp, 22), FKLF (H KLF11, 1.6 Kb, 3) or FGIF (750 bp, 23) were generated by RT-PCR using reaction conditions described below from total RNA isolated from K562 cells with forward and reverse primers containing a *BglII* site to allow ligation of the cDNA into the vector. Primer sequences were: NF-E4, 5'-GATACAATAAAGATCTCTGCCTCGTGTTGTCTGTTG-3' (forward), 5'-GATATATAGAAGATCTTTACCCTTGGCTCAGATGAA-3' (reverse), FKLF, 5'-

GAAGATCTCCTGCACGATGCACACG-3' (forward), 5'-AGATCTAGGCAGAGGCTGGCAT-3' (reverse), and FGIF, 5'-GATACAATAAAGATCTATGGAAAAAGAAA AAGGAAA-3' (forward), 5'-GATATATAGAAGATCTTTAAGACTGAGGT GAAGAAT-3' (reverse). The BglII sites are underlined. The 0.8 Kb *ApaI-HindIII* gg1-VP64 fragment described above was made blunt-ended and ligated into *BglII*-cut, blunt ended and phosphatased pμ'LCR-β pr-*BglII*-β int2-enh. These constructs were lipofected into CID-dependent wild-type β-YAC BMCs as described above.

Characterization of AP20187-dependent cells. Bone marrow- and fetal liver-derived cells were expanded and harvested at various times during culture for analysis of murine and human globin gene expression by RNAse protection (24), RT-PCR, or antibody staining (21) to detect globin chains.

For antibody staining, cells were washed with PBS in 15 ml conical tubes, then fixed in one ml freshly prepared 5% paraformaldehyde/PBS, pH 7.2, for one hour at 37°C. After addition of 10 ml PBS/0.1% BSA (PBS/BSA), cells were centrifuged at 200 x g for 5 minutes, resuspended in 0.5 ml methanol and incubated at room temperature for five minutes. Ten ml PBS/BSA were added; cells were centrifuged and washed once more in PBS/BSA. Cells were resuspended in 100 μl PBS/BSA containing 0.1% triton X-100 (PBT) and incubated for 30 minutes at room temperature with one μg primary mouse anti-human γ-globin chain antibody (Cortex catalog # CR8115M1, San Leandro, CA) diluted in PBT. 10 ml of PBS/BSA were added and cells were centrifuged as above. This wash was repeated twice more. Cells were resuspended in 100 μl PBT and incubated with 100 μl secondary FITC-labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, catalog #115-095-146, West Grove, PA) diluted 1:750 in PBT at room temperature for 30 minutes. Cells were washed in the same manner as for the primary antibody. Pellets were resuspended in 50 μl PBS; 5 μl aliquots were applied to slides. Control samples were prepared similarly except that the fix only control had no antibody added and the secondary antibody control had no primary antibody added. Results were observed with a Nikon E800 microscope at 200 x magnification using an EF-4 FITC Hyq filter set. Results were documented using a Photometrics Cool Snap ES camera and MetaMorph software. Software was set to automatically correct for non-specific background using an image of the secondary antibody control sample.

Analysis of globin and transcription factor mRNA levels using RT-PCR. Total cDNAs were synthesized from RNA isolated from β-YAC bone marrow or fetal liver cells using an oligo-dT primer (Promega, Madison, WI) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). One μg of total RNA was combined with 0.5 μg oligo-dT in a total volume of 11 μl and preheated at 70°C for 10 minutes. The reaction mixture was then cooled rapidly on ice followed by the addition of 4 μl 5X first strand buffer, 2 μl 0.1 M DTT, 1 μl 10 mM dNTP mix and 1 μl RNasin. The reaction mixture was heated to 42°C for 2 minutes, at which point 1 μl of Superscript II RT was added and the reaction was incubated at 42°C for 50 minutes. The RT enzyme was heat inactivated by incubation at 70°C.

For globin gene products, PCR was performed using the following three sets of primers in a single reaction: mouse α-globin, 5'-GATTCTGACAGACTCAGGAAGAAAC-3' (forward), 5'-CCTTTCCAGGGCTTCAGCTCCATAT-3' (reverse), human γ-globin, 5'-GACCGTTTTGGCAATCCATTTC-3' (forward), 5'-TATTGCTTGCAGAATAAGCC-3' (reverse), and human β-globin, 5'-ACACAACTGTGTTCACTAGCAACCTCA -3' (forward), 5'-GGTTGCCCATAACAGCATCAGGAGT-3' (reverse). Each reaction contained 5 μl 10X NH_4 buffer, 2 μl 50mM $MgCl_2$, 0.5 μl 25mM dNTP mix, 25 pmol forward primers, 25 pmol reverse primers, 2 μl cDNA, and 1 U Biolase Taq polymerase (Bioline, Randolph, MA). PCR was carried out with initial denaturation at 95°C for 7 minutes, 25 cycles of 1 minute steps at 95°C, 58°C, and 72°C, followed by a final extension at 72°C for 10 minutes. All globin primers were

designed to cross an exon so that RNA-templated reverse-transcribed DNA amplification products could be distinguished from gene amplification products.

Similar reaction conditions were used for FGIF and NF-B4 mRNAs; primer sets were the same as used to generate the full-length cDNA clones described above. RT-PCR for ggI-VP64 mRNA was performed as described, except that the first PCR was omitted (19). All of the PCR reactions to detect expression were semi-quantitative.

RESULTS AND DISCUSSION

We have previously described a system that allows multi-potential cell lines to be established from murine bone marrow. This system uses a retroviral vector to introduce a gene encoding a conditional signaling molecule into mouse BMCs, followed by activation of the signaling molecule using a small molecule drug called a chemical inducer of dimerization (25). A CID-activated derivative of the thrombopoietin receptor, mpl, induces transduced mouse BMCs to expand dramatically in culture. Cells generated in this manner adopt predominantly megakaryocytic features but also include multi-potential progenitors capable of generating monocytes, neutrophils and erythroid cells, but not B or T lymphoid cells upon addition of the appropriate growth factors (17, 26). Cultures can be maintained for longer than a year, and cell growth remains strictly dependent upon the continued presence of the CID. Our initial studies demonstrated that cells generated in this manner expressed adult mouse α-, β^{maj}- and β^{min}-globin mRNAs that could be readily detected by RNase protection assays (RPAs; data not shown).

To test whether the human β-globin locus would be appropriately regulated in cells derived from the bone marrow of adult mice, we generated F36Vmpl-transduced cells from transgenic mice containing a 155 Kb β-YAC. Three independent cell populations were established and assessed. RPAs demonstrated that the resultant CID-dependent cells expressed human β-globin mRNA, but not human γ-globin, establishing that proper developmental control was maintained (Figure 1, panel A). The higher level of human β-globin relative to mouse α-globin was attributed to a significant reduction in mouse α-globin levels in the cells compared to the β-YAC mice. A parallel decrease in mouse β-globin mRNA was also observed (data not shown). These findings also indicate a lack of environmental influence on the pattern of β-like globin gene expression, suggesting that the mechanisms regulating β-globin expression are cell-autonomous.

To confirm that the pattern of globin gene expression observed in β-YAC mice is maintained in derivative cell lines, we performed two additional studies. First, we examined the pattern of globin gene expression in cells derived from transgenic mice containing a 248 Kb -117 Greek HPFH β-YAC. In humans, a point mutation (G to A transversion) at position -117 relative to the mRNA start site of the $^{A}\gamma$-globin gene causes the Greek form of hereditary persistence of fetal hemoglobin (2). Previous studies showed that -117 $^{A}\gamma^{R}$ Greek HPFH β-YAC transgenic mice maintained human γ-globin expression into adult life (16). Similarly, three independent cell populations derived from the marrows of these mice expressed significant levels of γ-globin in addition to β-globin (Figure 1, panel A). The presence of human γ-globin chains in these cells was confirmed by fluorescent anti-γ-globin antibody staining (Figure 1, panel B). Although γ-globin chains were detected, no hemoglobin was formed in these multi-potential cells as assessed by benzidine staining.

As a second test of whether the pattern of β-like globin gene expression in β-YAC mice was reflected in the derived cells, we generated cell populations from the fetal livers of post-gestation day 12 wild-type β-YAC mice, which express significant levels of γ-globin. In three independently derived cell populations, human β-globin mRNA was detected by RT-PCR, whereas γ-globin mRNA was not expressed (data not shown). These results are consistent with either a switch from γ- to β-globin gene expression prior to or during establishment of cell culture, or alternatively, that fetal liver cells capable of extensive growth in the presence of F36Vmpl signaling are committed to the generation of erythroid progeny that express solely β-globin.

Reactivating γ-globin gene expression may be beneficial for patients with sickle cell anemia. BMCs derived from β-YAC mice may be useful for screening putative γ-globin inducers. Since 5-azacytidine (5-aza), an inhibitor of DNA methylation, is a strong inducer of γ-globin gene expression (27, 28), we tested whether 5-aza could activate γ-globin transcription in wild-type β-YAC BMCs. As shown in Figure 2, panel A, 5-aza induced significant levels of γ-globin transcription, thereby establishing that γ-globin gene expression can be induced in these cells. However, γ-globin expression was not induced by a number of other compounds, including α-aminobutyric acid, sodium butyrate, valproic acid, or trichostatin A (data not shown).

Next we tested an artificial transcription factor for its ability to induce γ-globin gene expression in wild-type β-YAC BMCs. We employed a synthetic construct, gg1-VP64, in which a zinc finger DNA binding domain, designed to target the region proximal to the -117 position of the γ-globin promoter, was linked to the VP64 transcriptional activator domain (19). This transcription factor has been shown to interact directly with the γ-globin promoter and to up-regulate γ-globin gene expression in K562 cells (19). A pool and five independent clones of β-YAC cells stably expressing gg1-VP64 displayed detectable levels of γ-globin mRNA by RT-PCR (Figure 2, panel B). The weak induction of γ-globin observed may be due to the relatively low expression of gg1-VP64 from the CMV promoter, which is inefficient in these cells.

Finally, the ability of previously identified fetal globin transcription factors to activate γ-globin gene expression was assessed. Full length cDNAs for each factor were placed under control of a human β-globin locus LCR/β-globin gene promoter cassette in a construct previously shown to confer erythroid-specific gene expression upon linked cDNAs (20, 21). FGIF, FKLF and gg1-VP64 under control of LCR β-globin promoter sequences reactivated γ-globin gene transcription in the wild-type CID-dependent β-YAC BMCs (Figure 3, Panel A), whereas NF-E4 did not. Expression of all the transactivator cDNAs was detected at the RNA level in the BMCs, except for FKLF, which was not tested (Figure 3, Panel B). The inability of NF-E4 to induce γ-globin gene expression in these cells was consistent with previous data demonstrating that it does not reactivate fetal globin synthesis in adult transgenic mice (29).

CID-dependent β-YAC cells may be useful primarily for identifying transcription factors that reactivate γ-globin gene expression, and to a lesser extent, for screening some chemical inducers of γ-globin expression for the treatment of sickle cell disease or β-thalassemia. γ-globin transcription went from a completely repressed state to detectable mRNA levels in all instances where expression was observed. This qualitative γ-globin "off-on" switch is unique to these cells; in other established cell lines only a change in constitutive expression may be measured. Although γ-globin was activated by 5-azacytidine (which affects methylation) or by an HPFH mutation, treatments that affect acetylation (butyric acid, etc.) did not induce γ-globin synthesis, suggesting that these cells do not completely mirror the erythroid phenotype. Alternately, regulation of human γ-globin in mice may be fundamentally different then it is in humans and these cell populations reveal that difference. However, gg1-VP64, FKLF and FGIF activation, coupled with the HPFH results, clearly demonstrates that these cells offer an ideal system to clone and characterize transcriptional activators that act directly upon the γ-globin gene promoters.

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FOOTNOTES

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'The abbreviations used are: LCR, locus control region; HS, DNase I-hypersensitive site; β-YAC, human β-globin locus yeast artificial chromosome; HbF, fetal hemoglobin, KLF, kruppel-like factor; BFUe, burst forming units-erythroid; MEL, mouse erythroleukemia cells; HFE, human fetal liver cells; BMC, bone marrow cells; CID, chemical inducer of dimerization; mpl, thrombopoietin; HPFH, hereditary persistence of fetal hemoglobin; 5-FU, 5-fluorouracil; DMEM, Dulbecco's Modified Eagle Medium; IRES, internal ribosomal entry site; IMDM, Iscove's Modified Dulbecco's Medium; FBS, fetal bovine serum; RPA, RNase protection assay; 5-aza, 5-azacytidine.

FIGURE LEGENDS

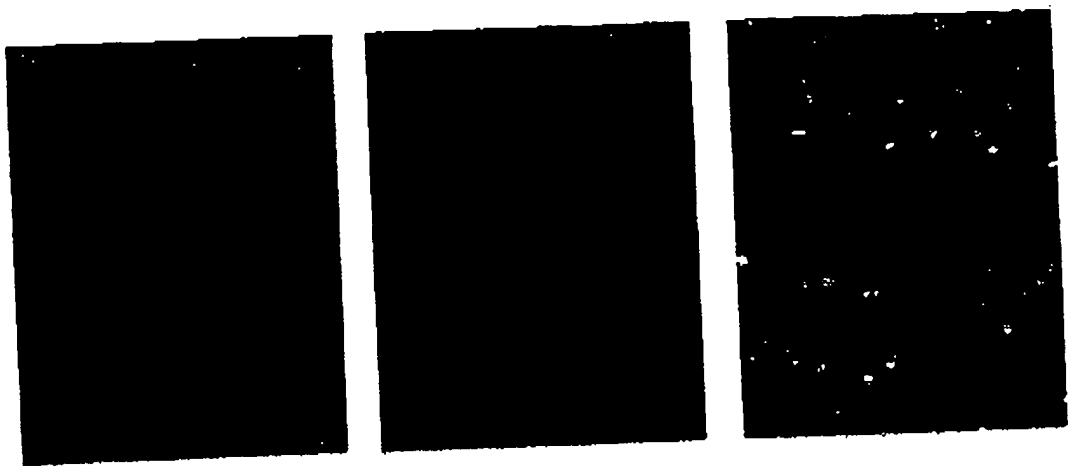
Figure 1. Panel A. Human β-globin transcripts are detected in CID-dependent cells derived from 155 Kb wild-type β-YAC transgenic mice and CID-dependent cells derived from 248 Kb -117 Greek HPFH β-YAC transgenic mice exhibit hereditary persistence of fetal hemoglobin (HPFH). The autoradiograph shows results of RNase protection analysis. Sample sources are illustrated above the panel; numbers indicate samples collected from more than one cell population or animal. Protected fragments are shown to the right of the panel; pBR322 *MspI* molecular weight markers are shown to the left. Hu β, human β-globin (205 bp); Hu γ, human γ-globin (170 bp); Mo α, mouse α-globin (128 bp), wt β-YAC, wild-type β-YAC transgenic mice; FL, fetal liver; Bl, blood. γ/(γ+β) quantitative values are the averages of two separately established cell populations; if more than two populations were analyzed standard deviations are also shown. Panel B. Fluorescent antibody staining of human γ-globin protein chains in CID-dependent wild-type or -117 HPFH β-YAC BMCs. γ-globin chains are detected in -117 HPFH β-YAC CID-dependent BMCs, but not in wild-type β-YAC BMCs. Upper) non-transgenic BMCs. Middle) wild-type β-YAC BMCs. Lower) -117 HPFH β-YAC BMCs. Background staining in the upper and middle panels is due to fixation and non-specific staining with the secondary antibody. After background correction, 30% of the cells in the lower panel are positive for γ-globin chain staining.

Figure 2. Panel A. 5-Azacytidine reactivates γ-globin mRNA synthesis in CID-dependent wild-type β-YAC bone marrow-derived cells. Five μg of total RNA was subjected to RNase protection analysis, as indicated above the autoradiograph. Sample names, treatment and concentrations of 5-Aza, when included, are shown above the autoradiograph; protected fragments are shown on the right side of the autoradiograph, molecular weight markers on the left. Abbreviations are as described in the legend to Figure 1. An overall decrease in globin mRNA production due to cytotoxicity was observed at 20 mM 5-Aza. γ/(γ+β) quantitative values are as described in Figure 1. Panel B. γ-globin expression is reactivated by gg1-VP64, a synthetic γ-globin-specific Zn finger activator protein, in CID-dependent wild-type β-YAC BMC pools and clones. Linearized pcDNA3.1/Hygro gg1-VP64 was lipofected into cells; clones were obtained from the transfected cell pool by limited dilution. Sample names are shown above the image, molecular weight markers to the left and RT-PCR products to the right. Abbreviations are as for Panel A. Expression of globin mRNAs in the pool or clones was assayed by RT-PCR (Upper). Expression of gg1-VP64 was assessed by RT-PCR (Lower) Hu β, 212 bp; Hu γ, 165 bp; Mo α, 122 bp; gg1-VP64, 300 bp.

Figure 3. Panel A. Fetal globin transcriptional activators induce γ-globin gene expression in CID-dependent wild-type β-YAC BMC pools. Linearized pμ'LCR-β pr-cDNA-β int2-enh constructs were lipofected into cells. Sample names and full-length cDNAs tested are shown above the image. Expression of globin mRNAs in the pools was assayed by RT-PCR. Labeling is as for Figure 2, Panel B.

Panel B. Expression of transcription factors was assessed by RT-PCR. Sample names are shown above the image. (-), non-transfected CID-dependent wild-type β-YAC BMCs; (+), CID-dependent wild-type β-YAC BMCs transfected with indicated cDNA clone. Molecular weight marker sizes are shown on the left side of the image and sizes of RT-PCR fragments are displayed on the right side. FGfF, 750 bp; NF-β4, 600 bp; gg1-VP64, 300 bp.

Blau et al., Figure 1



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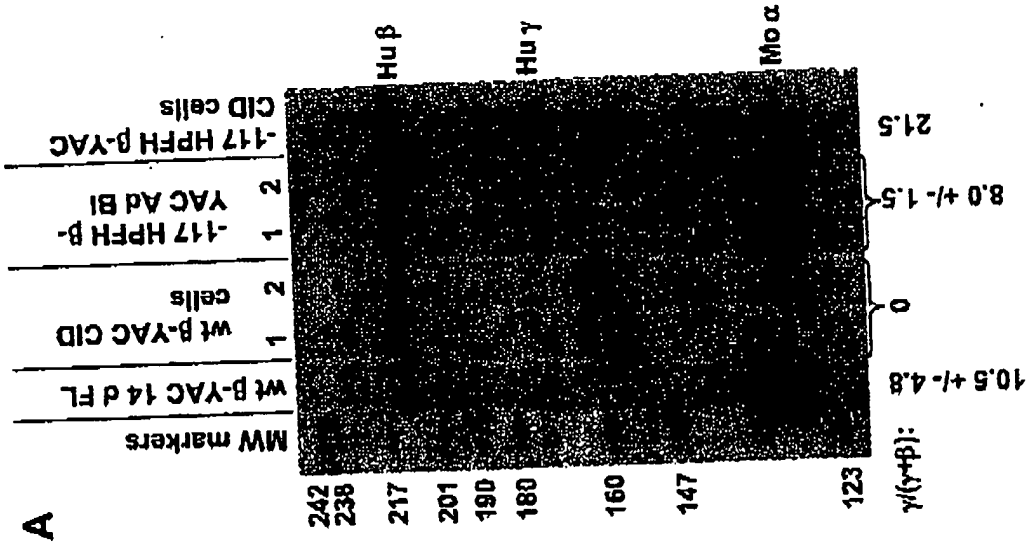

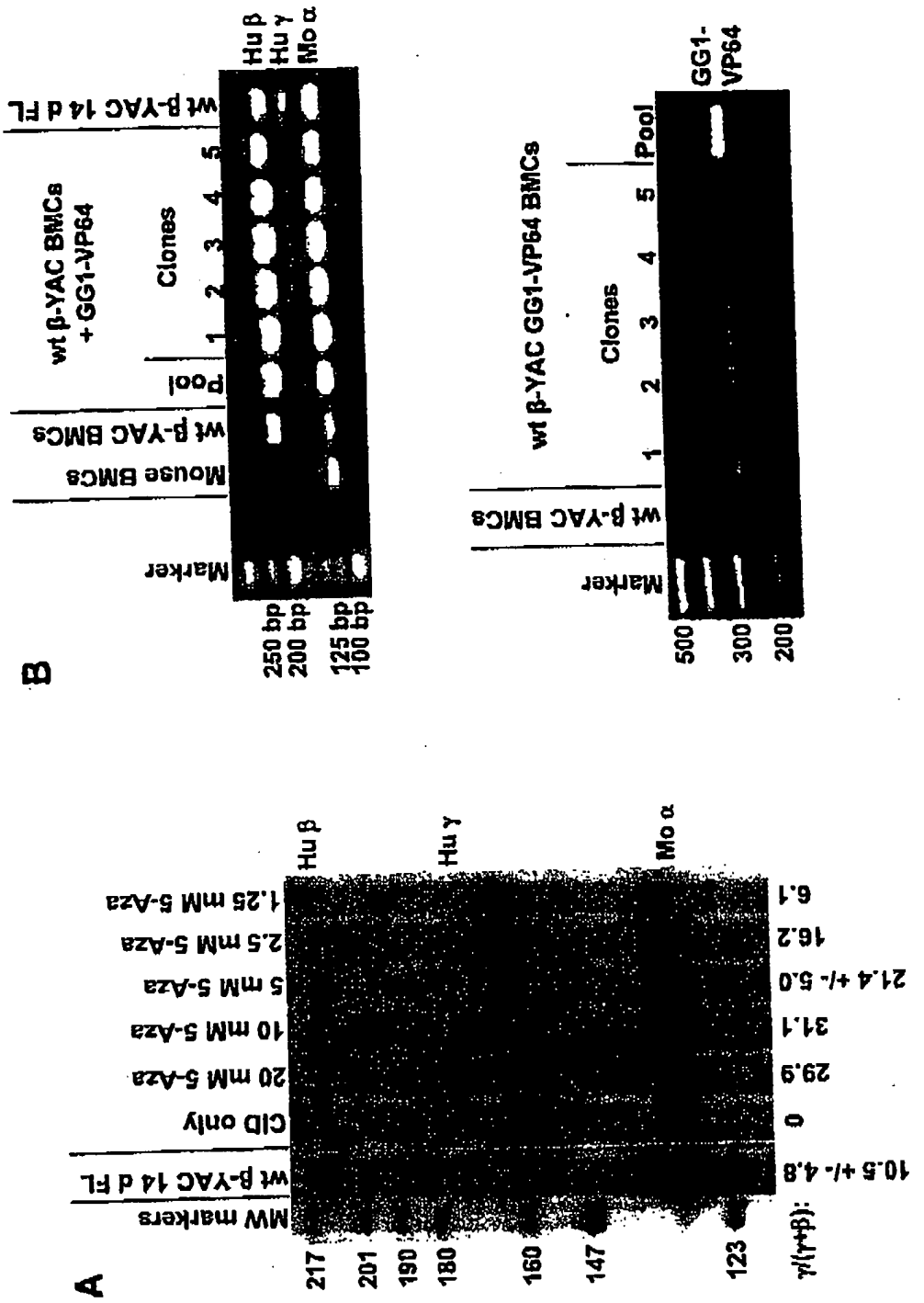

Blau et al., Figure 2

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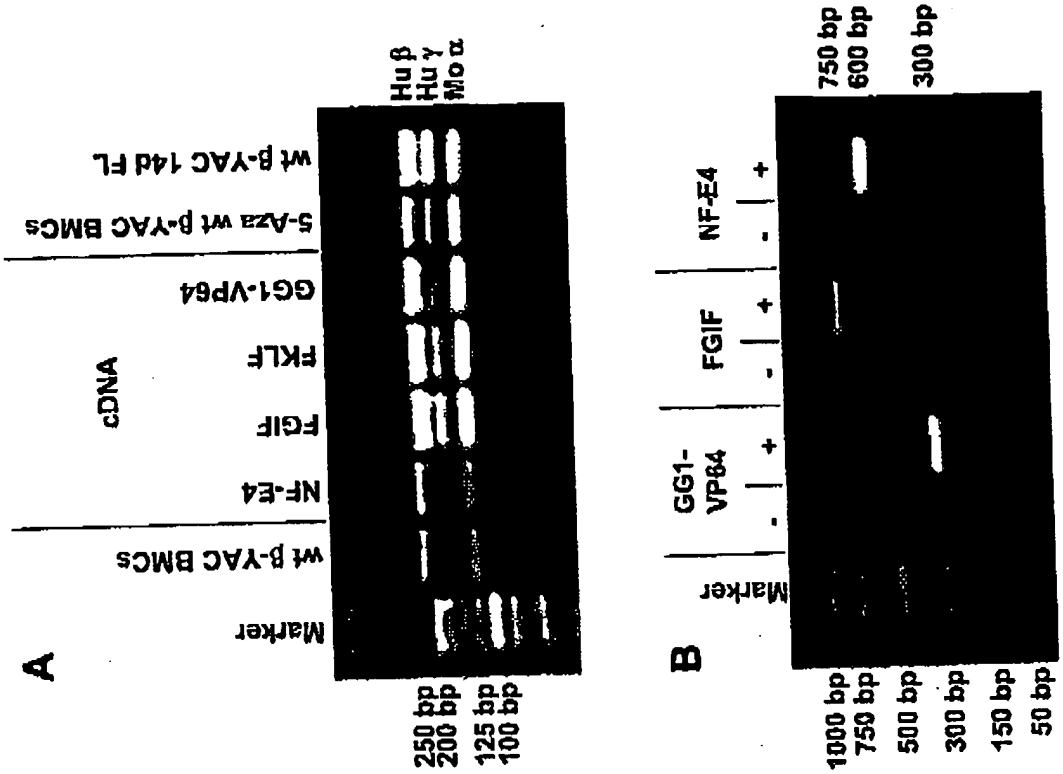
Blau et al., Figure 3



Sheet 1 of 2

Substitute Form PTO-1449 (Modified) — Department of Commerce, Patent and Trademark Office

Attorney's Docket No.	Application No.
17083-003002/1227B	09/586,625
Applicant: Carlos F. Barbas III et al.	
Filing Date: June 2, 2000	Group Art Unit: 1646

List of Patents and Publications for Applicant's Information Disclosure Statement

(37 CFR §1.98(b))

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	~~AA~~	~~2003/0143669~~	~~07/31/03~~	~~Genscore, Inc.~~	~~705~~	~~1~~	~~03/07/01~~
SHS	AB	2003/0186841	10/02/03	Barbas III et al.	514	1	04/23/03
	AC	2004/0224385	04/21/05	Barbas et al.	435	69.1	06/18/04
	AD	2005/0084885	04/11/05	Barbas, III et al.	435	6	09/14/04
	AE	2005/0148075	07/07/05	Barbas, C.F.	435	455	08/21/03
	AF	6,790,941	09/14/04	Barbas III et al.	530	400	02/09/00

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
SHS	AG	01/52620	07/26/01	PCT				
	AH	02/06463	01/24/02	PCT				
	AI	2002/097050	12/05/02	PCT				

Other Documents (Include Author, Title, Date, and Place of Publication)

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	AK	Beerli, R.R.and C.F. Barbas III, "Engineering polydactyl zinc-finger transcription factors," Nature Biotechnology 20(2): 135-41 (2002)
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	AO	Blau et al., "γ-globin gene expression in CID-dependent multi-potential cells established from beta-YAC transgenic mice," J. Biol. Chem. August 30, 2005
	AP	Dreier et al., "Development of zinc finger domains for recognition of the 5'-ANN-3' family of DNA sequences and their use in the construction of artificial transcription factors," J. Biol. Chem. 276(31): 29466-78 (2001)
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Examiner Signature	Date Considered

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Sheet 2 of 2

Substitute Form PTO-1449 (Modified) U.S. Department of Commerce Patent and Trademark Office	Attorney's Docket No. 17083-003002/1227B	Application No. 09/586,625
List of Patents and Publications for Applicant's Information Disclosure Statement	Applicant Carlos F. Barbas III et al.	
	Filing Date June 2, 2000	Group Art Unit 1646

(37 CFR §1.98(D))

Other Documents (Include Author, Title, Date, and Place of Publication)

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